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Evaluation of butylated hydroxyanisole, *myo*-inositol, curcumin, esculetin, resveratrol and lycopene as inhibitors of benzo[*a*]pyrene plus 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J mice

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Abstract

The potential activities of butylated hydroxyanisole (BHA), *myo*-inositol, curcumin, esculetin, resveratrol and lycopene-enriched tomato oleoresin (LTO) as chemopreventive agents against lung tumor induction in A/J mice by the tobacco smoke carcinogens benzo[*a*]pyrene (BaP) and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were evaluated. Groups of 20 A/J mice were treated weekly by gavage with a mixture of BaP and NNK (3 μmol each) for 8 weeks, then sacrificed 26 weeks after the first carcinogen treatment. Mice treated with BHA (20 or 40 μmol) by gavage 2 h before each dose of BaP and NNK had significantly reduced lung tumor multiplicity. Treatment with BHA (20 or 40 μmol) by gavage weekly or with dietary BHA (2000 ppm), curcumin (2000 ppm) or resveratrol (500 ppm) from 1 week after carcinogen treatment until termination had no effect on lung tumor multiplicity. Treatment with dietary *myo*-inositol (30 000 ppm) or esculetin (2000 ppm) from 1 week after carcinogen treatment until termination significantly reduced lung tumor multiplicity, with the effect of *myo*-inositol being significantly greater than that of esculetin. Treatment with dietary LTO (167, 1667 or 8333 ppm) from 1 week before carcinogen treatment until termination had no effect on lung tumor multiplicity. The results of this study demonstrate that BHA is an effective inhibitor of BaP plus NNK-induced lung tumorigenesis in A/J mice when administered during the period of carcinogen treatment and that, among the compounds tested, *myo*-inositol is most effective after carcinogen treatment.

Keywords: Butylated hydroxyanisole; *myo*-Inositol; Curcumin; Esculetin; Resveratrol; Lycopene; Benzo[*a*]pyrene; 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; Chemoprevention

1. Introduction

Lung cancer will kill over 160 000 people in the US in 1998, and over one million worldwide [1,2]. Approximately 85% of lung cancer is caused by smoking [3]. There are 47 million smokers in the

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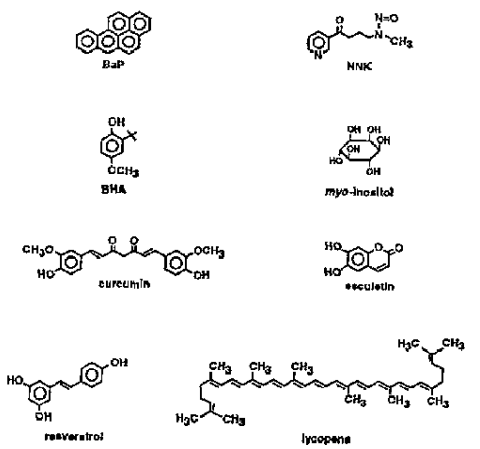

Fig. 1. Structures of the compounds used in this study.

US and one billion smokers worldwide [4,5], all at high risk for lung cancer. Moreover, there are 44 million ex-smokers in the US and their risk for lung cancer remains high for up to 10 years after cessation of smoking [4,6]. Chemoprevention is a way to decrease lung cancer risk in people exposed to cigarette smoke. Our goal is to discover and develop effective chemopreventive agents against lung carcinogens in tobacco smoke [7].

The major lung carcinogens in tobacco smoke are likely to be polycyclic aromatic hydrocarbons, typified by benzo[*a*]pyrene (BaP), and the tobacco-specific nitrosamine 4(methylnitrosamino)-1-(3pyridyl)-1-butanone (NNK) [7,8]. A mixture of BaP and NNK induces lung tumors in the A/J mouse and this is a convenient system for evaluating potential chemopreventive agents [9]. In the present study, we have used this model to determine the efficacy of several compounds during various stages of tumorigenesis by BaP plus NNK.

Butylated hydroxyanisole (BHA) is a common food additive which has chemopreventive activity in a number of systems, including inhibition of lung tumors in mice treated with either BaP or NNK [10–17]. In this study, we evaluated its activity when administered before or after BaP plus NNK. *myo*-Inositol occurs widely in foods and inhibits tumorigenesis in the mammary gland, colon, and lung [18–22]. Curcumin, the major yellow pigment of turmeric, is commonly used as a food additive [23]. It inhibits prostaglandin metabolism, inflammation, and induction of ornithine decarboxylase and is active as a chemopreventive agent against tumors of the oral cavity, colon, skin and other tissues [24–31]. Assays for its activity as an inhibitor of lung tumorigenesis have not been reported. Esculetin, a naturally occurring compound, inhibits the 5- and 12-lipoxygenase pathways of arachidonic acid metabolism [32] and mammary tumorigenesis in rats [33]. Resveratrol occurs in a variety of plant species including grapes, has a number of properties associated with chemoprevention, and inhibits mouse skin tumorigenesis in the promotion stage [34]. We evaluated *myo*-inositol, curcumin, esculetin, and resveratrol for chemopreventive activity by administration in the diet subsequent to treatment with BaP plus NNK. Lycopene is a carotenoid present in tomatoes. It inhibited the growth of several cancer cell lines in tissue culture studies [35]. Serum lycopene levels were inversely proportional to lung cancer incidence in one study, but not in another [36,37]. Lycopene inhibited lung tumorigenesis in male mice, but not in females, given a mixture of *N*-nitrosodiethylamine, *N*-methyl-*N*-nitrosourea and 1,2-dimethyl-hydrazine [38]. Lycopene-enriched tomato oleoresin (LTO) inhibited rat mammary and liver tumorigenesis, and tomato juice inhibited bladder tumorigenesis in rats [39–41]. Among carotenoids, lycopene has the highest antioxidant properties [42]. It is absorbed readily from traditional food sources and lowers oxidative damage [43,44]. We evaluated LTO for chemopreventive activity when given in the diet before, during and after treatment with BaP plus NNK. The structures of BaP, NNK, and the compounds tested for chemopreventive activity are shown in Fig. 1.

2. Materials and methods

2.1. Chemicals and test substances

BaP was obtained from Aldrich Chemical Co., Milwaukee, WI. NNK was synthesized [45]. Cottonseed oil, BHA (a mixture of 90% 3-*t*-butyl-4-hydroxyanisole and 10% 2-*t*-butyl-4-hydroxyanisole), *myo*-inositol, curcumin and resveratrol were purchased

from Sigma Chemical Co., St. Louis, MO. Esculetin was obtained from Fluka, Buchs, Switzerland. LTO, containing 5.9% lycopene, was obtained from LycoRed Natural Products Industries, Ltd., Beer-Sheva, Israel. All materials were used without further purification.

2.2. Stability of LTO in diet

LTO-containing diets were prepared every 3 weeks and stored at 4°C. Diets were added to the food containers in the mouse cages every 4 days. For analysis of lycopene in the diet, it was extracted from LTO-containing diets by sonication for 5 min with hexane/methylene chloride (5:1) containing 1.2 mM butylated hydroxytoluene. The mixture was centrifuged at 5000 rev./min for 10 min and the supernatant removed. The solvent was evaporated in the dark under a gentle stream of N_2, then redissolved in 30 μl methylene chloride for analysis by HPLC. HPLC analysis was performed on a 4.6 mm × 25 cm 10 μm C18 Vydac reverse phase column (The Separations Group, Hesperia, CA) with isocratic elution by 55% solvent A (1% methanol in H_2O) and 45% solvent B (methanol/acetonitrile/methylene chloride/H_2O, 10:4:1:0.16) at 1 ml/min, with UV detection (460 nm) [46]. The retention time of lycopene was 20 min.

2.3. A/J mouse tumorigenicity experiments

Treatment of mice with BaP plus NNK was carried out essentially as described [9]. Female A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The age requested was 5 weeks, but the weights of the mice 2 weeks later were not equivalent: in Experiments 1 and 2, the mean weights were 20.3 g and 19.0 g, respectively, while in Experiment 3, the mean weight was 16.1 g. Experiments 1 and 2 were carried out in the Research Animal Facility of the American Health Foundation. Experiment 3 was carried out in the specific pathogen-free animal quarters of the University of Minnesota Cancer Center. In Experiment 1, the mice were maintained on a modified AIN-76A diet, no. 112016 pellet form, from Dyets, Inc (Bethlehem, PA). This diet contains (g/kg): vitamin free casein, 200; DL-methionine, 3; cornstarch, 470; dyetrose corn starch, 50; dextrose, 130; cellulose, 50; corn oil, 50; salt mix, 35; vitamin mix, 10; choline bitartrate, 2. In Experiment 2, the powdered form of the same diet was used, with additions of the chemopreventive agents (ppm) as summarized in Table 1. In Experiment 3, the mice were maintained on an AIN-93G diet before and during carcinogen administration, and on an AIN-93M diet, starting 1 week after the last carcinogen administration. The AIN-93G/M diet is improved over the AIN-76A diet and we wanted to begin using it in our mouse lung tumorigenesis experiments [47]. The AIN-93G diet (Dyets no. 110700) contains (g/kg): cornstarch, 397.5; casein, 200; dextrinized cornstarch, 132; sucrose, 100; soybean oil, 70; cellulose, 50; mineral mix no. 210025, 35; vitamin mix, 10; L-cystine, 3; choline bitartrate, 2.5; *t*-butyl-hydroquinone, 0.014 [47]. The AIN-93M diet (Dyets no. 110900) contains (g/kg): cornstarch, 465.7; casein, 140; dextrinized cornstarch, 155; sucrose, 100; soybean oil, 40; cellulose, 50; mineral mix no. 210050, 35; vitamin mix, 10; L-cystine, 1.8; choline bitartrate, 2.5; *t*-butyl-hydroquinone, 0.008 [47]. One group was maintained on a modified AIN-76A diet throughout the experiment for comparison. All diets in Experiment 3 were powdered.

In Experiment 3, the powdered diet was fed from metal boxfeeders (Lab Products, Inc., Seaford, DE), no. 30604. This device minimized waste of diet and allowed close monitoring of food consumption.

In all experiments, carcinogen treatment commenced at an apparent age of 7 weeks. Each mouse was treated by gavage with a mixture of BaP (3 μmol) and NNK (3 μmol) dissolved in 0.1 ml cottonseed oil. This was repeated weekly for a total of eight doses. The experiments were terminated 26 weeks after the first carcinogen dose.

In Experiment 1, group 1 was treated only with BaP and NNK in cottonseed oil. Group 2 was treated with 0.1 ml cottonseed oil 2 h before each dose of BaP and NNK in cottonseed oil. Groups 3 and 4 were treated with 0.1 ml cottonseed oil containing 20 μmol or 40 μmol BHA, 2 h before each dose of BaP and NNK in cottonseed oil. Groups 5 and 6 were treated with 0.1 ml cottonseed oil 2 h before BaP and NNK in cottonseed oil, then by gavage with either 20 μmol or 40 μmol BHA in 0.1 ml cottonseed oil, weekly from 1 week after the final dose of BaP and NNK until termination, 26 weeks after the first dose of BaP and NNK. Group 7 was untreated.

Table 1
Effects of BHA, *myo*-inositol, curcumin, esculetin, resveratrol, and LTO on lung tumorigenesis induced by BaP plus NNK in A/J mice[a]

Experiment	Group	Treatment	No. of mice at termination	Mean body weight		Lung tumors	
				After carcinogen treatment[b]	At termination	% of mice with tumors	Tumors per mouse ± SD
1	1	BaP + NNK	20	23.6	26.9	100	22.3 ± 8.5
	2	Cottonseed oil, then BaP + NNK	20	24.5	28.8	100	21.8 ± 6.5
	3	BHA (20 μmol), then BaP + NNK	20	23.6	28.0	100	6.5 ± 2.5[c]
	4	BHA (40 μmol), then BaP + NNK	20	23.0	28.0	90	4.6 ± 3.0[c,d]
	5	Cottonseed oil, then BaP + NNK, then BHA (20 μmol)	20	24.4	27.4	100	23.9 ± 10.2
	6	Cottonseed oil, then BaP + NNK, then BHA (40 μmol)	18	24.0	27.8	100	18.7 ± 9.2
	7	None	20	25.7	30.3	15	0.3 ± 0.8
2	1	BaP + NNK	20	20.4	25.7	100	25.2 ± 12.2
	2	BaP + NNK, then BHA (2000 ppm)	20	22.0	25.4	100	22.3 ± 7.8
	3	BaP + NNK, then myo-inositol (30 000 ppm)	20	21.7	26.3	100	10.3 ± 5.7[e,f]
	4	BaP + NNK, then curcumin (2000 ppm)	20	21.4	27.1	100	21.0 ± 6.1
	5	BaP + NNK, then esculetin (2000 ppm)	20	22.4	27.2	100	18.4 ± 9.1[g]
3	1	BaP + NNK (AIN-76A diet)	17	19.4	24.7	100	25.1 ± 9.0
	2	BaP + NNK (AIN-93G/Mdiet)	20	18.2	23.2	100	22.0 ± 9.4
	3	BaP + NNK, then resveratrol (500 ppm)	16	18.7	26.3	100	24.6 ± 10.9
	4	BaP + NNK, LTO throughout (185 ppm)	16	19.6	25.6	100	26.8 ± 11.4
	5	BaP + NNK, LTO throughout (1850 ppm)	16	19.1	24.9	100	25.5 ± 9.4
	6	BaP + NNK, LTO throughout (9260 ppm)	20	18.6	23.8	100	24.1 ± 7.9
	7	Cottonseed oil only	19	19.5	24.0	21	0.3 ± 0.6

[a] Female A/J mice were treated with eight weekly gavage doses of BaP + NNK (3 μmol of each) in 0.1 ml cottonseed oil. Chemopreventive agents were administered at various stages. In Experiment 1, BHA in cottonseed oil was given by gavage 2 h before each dose of BaP + NNK (groups 3 and 4), or weekly starting 1 week after BaP + NNK until termination 26 weeks after the first dose of BaP + NNK (groups 5 and 6). In Experiment 2, chemopreventive agents were given in the diet (groups 2–5), starting 1 week after BaP + NNK until termination 26 weeks after the first dose of BaP + NNK. In Experiment 3, the effects of the AIN-76A and AIN-93G/M diets were compared (groups 1 and 2). Resveratrol was given in the diet from 1 week after BaP + NNK until termination 26 weeks after the first dose of BaP + NNK (group 3). LTO was given in the diet from 1 week before BaP + NNK until termination 26 weeks after the first dose of BaP + NNK (groups 4–6).

[b] One week after final BaP + NNK dose

[c] Significantly less than groups 1 and 2, $P < 0.0001$.

[d] Significantly less than group 3, $P < 0.05$.

[e] Significantly less than group 1, $P < 0.0001$.

[f] Significantly less than all other groups, $P < 0.05$.

[g] Significantly less than group 1, $P < 0.02$.

In Experiment 2, group 1 was treated only with BaP and NNK in cottonseed oil. Groups 2–5 were treated with BaP and NNK as in group 1, with additions to the diet starting 1 week after the final dose of BaP and NNK. The mice in groups 2–5 were maintained on diets containing BHA (2000 ppm), *myo*-inositol (30 000 ppm), curcumin (2000 ppm) or esculetin (2000 ppm) until termination 26 weeks after the first dose of BaP and NNK.

In Experiment 3, groups 1 and 2 received only BaP and NNK in cottonseed oil, but were maintained on an AIN-76A modified or AIN-93G/M diet as described above. Group 3 received BaP and NNK in cottonseed oil, then resveratrol (500 ppm) in the diet, starting 1 week after the final dose of BaP and NNK until termination 26 weeks after the first dose of BaP and NNK. Groups 4–6 received LTO in the diet (185 ppm (10.9 ppm lycopene in diet), group 4; 1850 ppm (109 ppm lycopene in diet), group 5; 9260 ppm (546 ppm lycopene in diet), group 6) from 1 week before carcinogen treatment until termination of the experiment. Group 7 was treated with cottonseed oil only, 0.1 ml by gavage each week for 8 weeks, starting at age 7 weeks. Mean food consumption was 2.3 ± 0.3 g/day per mouse.

Lung tumors were counted at termination. Statistical comparisons of tumor multiplicity between control and treated groups within each experiment were carried out using one-way analysis of variance (ANOVA) with a priori contrasts for pairwise comparisons of interest.

3. Results

The results are summarized Table 1. None of the treatments with chemopreventive agents affected weight gain or survival of the mice. Cottonseed oil administered before BaP plus NNK had no effect on lung tumor incidence or multiplicity (Experiment 1, groups 1 and 2).

BHA administered in cottonseed oil 2 h before each dose of BaP plus NNK significantly reduced lung tumor multiplicity from 21.8 ± 6.5 to 6.5 ± 2.5 in the mice treated with 20 μmol BHA, and further to 4.6 ± 3.0 in the mice treated with 40 μmol BHA (Experiment 1, groups 3 and 4). However, weekly administration of BHA by gavage starting 1 week after carcinogen treatment had no effect on lung tumor multiplicity (groups 5 and 6). There were no effects on lung tumor incidence in Experiment 1.

In Experiment 2, dietary BHA or curcumin administered starting 1 week after eight weekly doses of BaP plus NNK had no effect on lung tumor multiplicity (groups 2 and 4). *myo*-Inositol administration after BaP plus NNK significantly inhibited lung tumor multiplicity, reducing the number of tumors per mouse from 25.2 ± 12.2 to 10.3 ± 5.7 (group 3). Esculetin had a small, but significant, effect on lung tumor multiplicity (group 5). There were no effects on lung tumor incidence.

In Experiment 3, mice maintained on the AIN-93G/M diet and treated with BaP plus NNK had the same lung tumor multiplicity as BaP plus NNK-treated mice maintained on the AIN-76A diet (groups 1 and 2). Lycopene, administered as LTO, was stable in the diet at 4°C for at least 3 weeks and at room temperature for at least 11 days, according to analysis by HPLC. Neither LTO given before, during and after BaP plus NNK treatment, nor resveratrol, given in the diet starting 1 week after BaP plus NNK administration, had any effect on lung tumor multiplicity or incidence (groups 3–6).

Mice in these experiments also developed forestomach tumors. In some animals, these were too numerous to count, consistent with previous results at this dose [9]. Therefore, no attempt was made to quantify these results. Only BHA given 2 h before BaP plus NNK appeared to inhibit forestomach tumor multiplicity.

4. Discussion

The strongest inhibition of lung tumorigenesis in this study was obtained by concurrent administration of BHA with BaP plus NNK. This resulted in a highly significant reduction of lung tumor multiplicity at both doses. These results are consistent with those obtained in previous studies of BHA as an inhibitor of lung tumorigenesis by either BaP or NNK. Wattenberg demonstrated strong inhibition of BaP-induced lung tumor multiplicity in A/J mice treated with BHA (42 or 83 μmol), 4 h before each of three doses of 12 μmol of BaP [14]. Pepin et al. showed that, in A/J mice, dietary BHA (5000 ppm) strongly inhibited

lung tumor induction by NNK given in the drinking water (total dose 43 μmol) [12]. BHA decreases DNA adduct formation in mouse lung by BaP [48,49]. This is believed to result from favorable modification of phase I and phase II metabolism of BaP, although the role of specific metabolic pathways has not been clearly delineated [14,17,48–54]. Studies on the effects of BHA on NNK metabolism have not been reported [8].

Witschi and Doherty showed that BHA, given in the diet (7500 ppm) starting 24 h after treatment of A/J mice with 24 μmol BaP, significantly reduced lung tumor multiplicity from 13.6 ± 1.8 to 8.3 ± 1.6 [16]. We did not observe any effect of BHA on lung tumorigenesis by BaP plus NNK, when it was given either by gavage or in the diet starting 1 week after carcinogen administration. Our dose of dietary BHA was lower than that used in the previous study and it was started 1 week rather than 1 day after carcinogen treatment. These differences in protocols may account for the different results. Based on the available data, BHA does not appear to be a very effective inhibitor of lung tumorigenesis when given subsequent to carcinogen treatment.

BHA causes forestomach tumors in rodents when administered in the diet at higher doses (0.52%) and for longer periods of time than those used here [10,11]. Although BHA is considered to be a rodent forestomach tumorigen, its potential as a human carcinogen is unclear [10,11]. The data obtained in Experiment 1, as well as previously published data, indicate that BHA and related analogues deserve further attention as potential chemopreventive agents.

Among the compounds administered after BaP plus NNK, *myo*-inositol showed the greatest chemopreventive activity. The dose of *myo*-inositol was chosen based on a previous study in which the same dose inhibited BaP-induced lung tumorigenesis, when given in the post-initiation period [21]. A subsequent study showed that *myo*-inositol (1% in the diet) was also effective in reducing BaP-induced lung tumor multiplicity when given either after the carcinogen or when administered before, during, and after treatment with either BaP or NNK [22]. The present results are entirely consistent with these studies. Although these doses of *myo*-inositol are high, there was no evidence of toxicity in this or previous studies. The mechanism of the *myo*-inositol chemopreventive effect is not known. However, inositol hexaphosphate, which can be converted to *myo*-inositol in vivo, inhibits phosphatidylinositol-3 kinase activity, activator protein-1 activation, and tumor promoter-induced cell transformation [55].

Esculetin showed limited inhibitory activity, but this was statistically significant. In a previous study, neither dietary esculetin (2300 ppm) nor its glucose conjugate esculin (4600 ppm) given before, during, and after NNK treatment had any effect on lung tumor multiplicity in A/J mice [56]. However, esculin strongly inhibited BaP tumorigenesis on mouse skin [57]. The effects of esculetin in the BaP plus NNK model require further investigation. There was no evidence for chemopreventive activity of LTO in our experiments. Curcumin and resveratrol were inactive in the post-initiation stage of carcinogenesis. Since these agents are considered to have antioxidant activity among other properties, these results do not support the role of oxidative damage in carcinogenesis by BaP plus NNK in this mouse lung tumor model.

In summary, the results presented here support further development of BHA and *myo*-inositol as chemopreventive agents against lung cancer. BHA would be developed for use in current smokers while *myo*-inositol could potentially be used for chemoprevention in ex-smokers. The effects of esculetin require further investigation, while no modification of tumor development was apparent in the experiments with curcumin, resveratrol, or LTO.

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